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Comparative study of protease activity of psychotrophic and mesophilic bacteria

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Abstract

Six bacterial colonies were isolated from solid agar medium containing skimmed milk from Ice sample from juice corner (Lucknow). Among these three isolates were found to be capable of producing protease alkaline pH and at different temperature ranging from 20-45 ° C. On the basis of larger clear zone formation at low temperature, i.e. 20 ° C, these three isolates was selected as potent protease producer strain, which are gram positive designated as BBAU/P-1, BBAU/P-2, BBAU/P-3. Eight bacterial colonies were isolated from skimmed milk agar from Soil sample of Garden. Among these four isolates were found to be capable of producing protease at different temperature, On the basis of larger clear zone at high temperature, i.e. 45 ° C. These four isolates designated as BBAU/P-4, BBAU/P-5, BBAU/P-6, and BBAU/P-7. Out of these seven protease producing bacteria, BBAU/P-5 produced high amount of protein as assayed by the cup-plate method based on zone hydrolysis around the well in solid media.Thus, results suggestmesophilic bacteria has more potential to produce protease as compare to psychotrophic bacteria.

Key-Words: Psychotrophicss, Mesophilic and Protease

Introduction

Proteases form a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganism, plant and animal. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (1). Bacteria belonging to Bacillus sp. are by far the most important source of several commercial microbial enzymes (2; 3; 4; 5; 6; 7; 8; 1; 9). Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (10; 7).*Bacillus* produces a wide variety of extra-cellular enzymes, including proteases.

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Several *Bacillus* species involved in protease production are e.g. *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis*(11; 4; 7; 12; 13 and 14)Cold environments represent a large proportion of Earth's area, including the Arctic, the Antarctic, oceans, and mountain areas (15). There have been a few reports on protease producing Arctic bacteria (16; 17). The protease released by Arctic bacteria showed optimal activity at low temperatures (18). Therefore, the Arctic region can be a good source offering cold-active enzymes. Cold-adapted microorganisms (i.e. psychrophilic or psychrotolerant bacteria) have adapted to cold habitats, making them valuable sources for cold-active enzymes with potential industrial applications. These organisms survive and grow well due to unique molecular and physiological adaptations despite the strong negative effect of low temperatures on biochemical reactions. These adaptations include increased structural flexibility of enzymes, unique lipid constituents of cell membranes, and rapid synthesis of cryoprotectants and cold-shock proteins (19; 15). Cold-active enzymes secreted by these microorganisms exhibit high catalytic efficiency at low and moderate temperatures, and are easily inactivated by a moderate increase in temperature (19).Interest in the enzymes produced by cold-adapted

microbial strains has recently increased (20; 21). However, most studies have focused on the properties of these enzymes (22;23) and few have investigated the regulation of their production according to the physiology of the microorganism (24;25;21).In our study, we are observing the protease activity of psychrophilic bacteria from ice of juice corner and the mesophilic bacteria of garden soil.

Material and Methods

Sampling

Sample taken from soil and ice for study were collected from two places of Lucknow in sterile plastic bag transported on freeze at 4°C. The two places are as follows:

- **Juice corner rajnikhand (sample A from ice)**
- **BBAU campus (sample B from soil)**

Isolation of protease producing bacteria

One gram of soil sample was collected from Garden Soil at BBAU Campus and one ml of cold water was collected from Juice corner at Rajnikhand and dissolved in 10ml of distilled water, marked as 10^{-2} dilution. The soil suspensions were serially diluted up to 10^{-7}. An aliquot of 0.1ml was drawn from 10^{-2} and 10^{-6} dilutions and streaked into sterile petriplates. The plates were incubated at 28-37°C for 24 hours.

Screening of the isolates for proteolytic activity

Individual bacterial colonies isolated above were further screened for proteolytic activity on Skim milk agar medium**(4)**

The pH of the medium was adjusted at 10 using 1N NaOH. Individual colonies were spot inoculated and were incubated at 30°C for 24 hours.

Identification of bacteria

Identification of high protease producing isolates was done on the basis of cell morphology, cultural characteristics and biochemical reactions as described in Bergey's manual of Systematic Bacteriology.

Secondary screening of protease producing bacteria

To detect any false positive strain proteolytic strain, each isolate was once again stabbed at the centre of the skim milk agar and examined for clear zone of hydrolysis around the colonies after incubation at 30^0 C and 37^0 C for 5 days.

Cultivation of microorganism

To study the growth of the organisms and protease secretion, a 250ml. Erlenmeyer flask containing 100 ml of Luria-Bertani broth [Containing (g/l)]Bacto-tryptone, 10, yeast extract, 5, NaCl, 10, and casein 10 or gelatine 5, pH adjusted to 7.0] was inoculated with 2-3 pure fresh colonies and incubated at 30^0 C, for 9 days. Five ml. of each culture solution was taken at various time intervals and used for assaying bacterial growth and proteolytic activity. Growth was assessed colorimeterically at 620 nm, a value of A 620=1 corresponds to 6×10^9 cells/ml. (26). The sample was centrifuged at 10,000 rpm for 10 min. and supernatant thus obtained was used for assays of proteolytic activity with gelatine or casein as the substrate using Cup Plate Method.

Protease assay by Dingle's cup plate method

Protease activity was assayed by Dingle's cup plate method (27) using agar plates (comprised of 1% (w/v) gelatine, 2.5% (w/v) agar, 0.01% (w/v) sodium azide as preservatives dissolved in potassium phosphate buffer (0.1 M, pH 7). Three identical circular wells were made in each plate with a cork borer. Agar plugs were removed and 100 µl of the crude was loaded into the each well. The plates were incubated at 37^0 C for 24 hrs in upright position. The clear zone of hydrolysis around the well was measured after flooding with developing solution containing of 15 gm $HgCl_2$ 20 ml concentrated HCl in 100 ml distilled water. Enzyme activity was expressed as zone of hydrolysis in mm.

Results and Discussion

Six bacterial colonies were isolated from solid agar medium containing skimmed milk from Ice sample from juice corner (Rajnikhand). Among these three isolates were found to be capable of producing protease alkaline pH and at different temperature ranging from 20-45 ° C. On the basis of larger clear zone formation at low temperature, i.e. 20 ° C, these three isolates was selected as potent protease producer strain, designated as BBAU/P-1, BBAU/P-2, BBAU/P-3 and has taken for further studies. & Eight bacterial colonies were isolated from skimmed milk agar from Soil sample from Garden soil (BBAU CAMPUS). Among these four isolates were found to be capable of producing protease at different temperature, On the basis of larger clear zone at high temperatue, i.e. 45 ° C. These four isolates designated as BBAU/P-4, BBAU/P-5, BBAU/P-6, BBAU/P-7.

The present study showed that the isolated bacteria from ice grow at low temperature and from soil grow at medium temperature. The morphology and characterstic of isolated bacteria showed rod or cocci shape, gram positive or negative, nearly two are nonmotile and five are motile. Colonies are usually Yellow or white in color. The isolated bacteria are basically psychrotrophic and alkalophilic in nature and showed different growth pattern under different environmental condition. Due to psychrotrophic nature bacteria prefer optimum temperature i.e, 15 to 30 °C. and Alkalophylic nature bacteria prefer optimum temperature i.e, 30 to 45 °C. When a bacteria grown on different pH it showed better growth on neutral pH.The

optimum temperature for an extracellular protease produced by Flavobacteriumpsychrophilum was at temperature between 25 and 40 ° C. In addition to that, the optimum temperature for protease production was between 30 and 45 ºC (28). Jobin and Grenier2003 (29) investigated the production of proteases by *Streptococcus suis*serotype 2 and recoded that the optimum temperature for protease production ranged from 25 to 42 ºC. Our results indicate that the optimum temperature for protease production ranged from 20 to 45 ºC. Maximum protease production was achieved at pH 9.0 by isolates. The production of protease increased as pH of the medium increases and reaches maximum at pH 9.0. After pH 9.0 there was a decrease in enzyme production. Results suggest that there is a stimulation of enzyme production at alkaline pH. The obtained results coincide with Kumar et al. 2002 (3) who have reported that protease production was maximum at pH 7 and 9 for *Bacillus* sp. According to found area of clear zone the maximum protease activity was found in BBAU/P-5 strain.

Conclusion

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Selection of the right organism plays a key role in high yield of desirable enzymes. Habitats that contain protein are the best sources to isolate the proteolytic microorganism. Proteolytic bacteria which are both mesophilic and psychrophilic, contain large number of bacteria.

Protease enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzymes recommendable. Thus, the search for new microbial sources is a continual exercise, but one must respect biodiversity.

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Table 1: Isolation of protease producing bacteria

Three strains were isolated from Sample A and Four strain were isolated from Sample B.

Sample	Characterisation	Strain
Sample A	Ice	BBAU/P - 1
		BBAU/P – 2
		BBAU/P – 3
Sample B	Soil	BBAU/P – 4
		BBAU/P – 5
		BBAU/P – 6
		BBAU/P – 7

Table 2: Morphological Characteristics

Morphological Characterstics	Sample A			Sample B			
	BBAU/P-1	BBAU/P-2	BBAU/P-3	BBAU/P-4	BBAU/P-5	BBAU/P-6	BBAU/P-7
Cell Shape	Cocci	Rod	Rod	Cocci	Cocci	Rod	Rod
Colony color	Yellow	Yellow	Light Yellow	White	White	White	White

Table 3: Gram Staining

Stain	BBAU/P-1	BBAU/P-2	BBAU/P-3	BBAU/P-4	BBAU/P-5	BBAU/P-6	BBAU/P-7
Gram Stain	+ve	+ve	+ve	-ve	+ve	+ve	+ve

Table 4: Biochemical Characterisation

Biochemical Characterisation	BBAU/P-1	BBAU/P-2	BBAU/P-3	BBAU/P-4	BBAU/P-5	BBAU/P-6	BBAU/P-7
Casein hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Motility Test	Nonmotile	Nonmotile	Motile	Motile	Motile	Motile	Motile
Catalase Test	+ve	+ve	+ve	-ve	+ve	+ve	+ve
Methyl Red Test	+ve	-ve	+ve	-ve	-ve	+ve	+ve
Citrate Utilization	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Mannitol Fermentation	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Lactose Fermentation	-ve	-ve	-ve	+ve	+ve	+ve	+ve
Sucrose Fermentation	-ve	-ve	-ve	+ve	+ve	+ve	+ve
Amylase Test	-ve	-ve	-ve	-ve	+ve	+ve	+ve

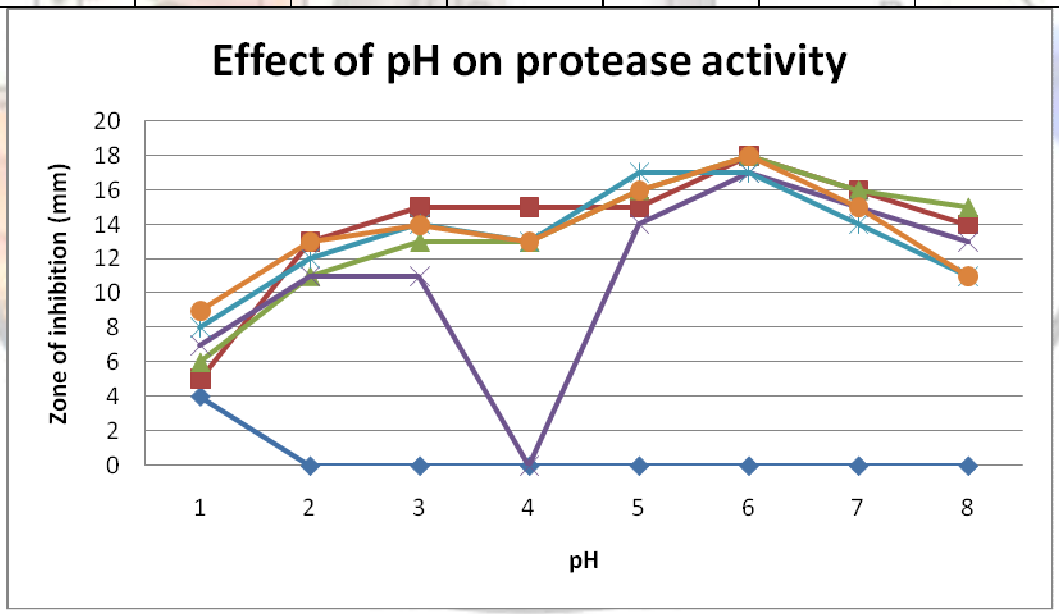

Table 5: protease activity shown by isolated bacteria

Isolated bacteria	Times in days	Zone of hydrolysis (mm)
BBAU/P – 1	2	10
	3	11
	4	12
	5	11
	6	11
	7	11
BBAU/P – 2	2	12
	3	13
	4	14
	5	14
	6	13
	7	13
BBAU/P – 3	2	12
	3	12
	4	13
	5	13
	6	14
	7	14
BBAU/P – 4	2	14
	3	14
	4	15
	5	16
	6	16
	7	15
BBAU/P – 5	2	16
	3	16
	4	18
	5	18
	6	17
	7	17
BBAU/P – 6	2	15
	3	15
	4	16
	5	16
	6	17
	7	16
BBAU/P – 7	2	14
	3	13
	4	15
	5	15
	6	14
	7	15

Table 6: protease activity shown by isolated bacteria in different time duration

Isolated bacteria	Times in days	Absorbance /5ml at 620 nm
BBAU/P – 1	2	0.1
	3	0.2
	4	0.25
	5	0.4
	6	0.5

	7	0.6
BBAU/P – 2	2	0.2
	3	0.3
	4	0.4
	5	0.5
	6	0.5
	7	0.6
BBAU/P – 3	2	0.4
	3	0.5
	4	0.7
	5	0.8
	6	1.0
	7	1.2
BBAU/P – 4	2	0.5
	3	0.6
	4	0.7
	5	0.8
	6	0.9
	7	1.2
BBAU/P – 5	2	0.8
	3	0.9
	4	1.1
	5	1.2
	6	1.3
	7	1.4
BBAU/P – 6	2	0.6
	3	0.7
	4	0.7
	5	0.8
	6	0.9
	7	1.1
BBAU/P – 7	2	0.7
	3	0.8
	4	0.8
	5	0.9
	6	1.0
	7	1.2